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An Electron Microscopic Investigation of Bovine Abortion

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AN ELECTRON MICROSCOPIC INVESTIGATION OF BOVINE ABORTION

BY

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OF BOVINE ABORTION

This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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Introduction

Bovine abortion continues to cause economic loss to farmers and ranchers. In South Dakota, monetary loss from bovine abortion has been estimated at 4 to 4.8 million dollars annually. (31) Brucellosis was once considered the major cause of bovine abortion. Since the prevalence of brucellosis has been reduced to less than 1% of the total United States cattle population, there has been an increase in demand for laboratory assistance in the diagnosis of the causes of bovine abortion. With the present day technology in microbiology, histology, and serology, a confirmed identification of the causative agent of bovine abortion can be made in only 30%-40% of cases examined. Results of serologic and histologic examinations seem to indicate infection occurs in numerous cases in which no etiologic agent can be identified. The search continues for methods which would increase the accuracy of laboratory diagnosis of the causes of bovine abortion.

Negative stain electron microscopy (EM) has been used to demonstrate viral structures in infected cell cultures, feces, and other clinical material. This technique was applied to lung and liver tissue extracts obtained from 500 aborted or stillborn calves submitted for diagnostic purposes to the South Dakota Animal Disease Research and Diagnostic Laboratory. The following goals were set for the project: 1. To determine if this technique can be used on fetal tissue to identify known etiologic agents of bovine abortion and compare results with those obtained by conventional techniques. 2. To determine if viral agents not detected by conventional means are present in tissues of aborted calves.

3. To attempt to isolate and identify any agents found, and 4. to determine if this technique has application in the routine diagnosis of bovine abortion.

Literature Review

Prior to 1950 there was a belief that if brucellosis were controlled, bovine abortion could be greatly reduced or even eliminated. During the past 25 years the prevalance of brucellosis has been reduced to less than 1% of the total United States cattle population, but bovine abortion continues to cause major economic loss. (31) Results of surveys in the United States, (4,19,31) Canada, (39,40) and New Zealand, (32) show that the cause of bovine abortion is not identified in approximately 70% of the cases examined. Inadequate observation and lack of records make it difficult if not impossible to obtain an accurate estimate of the abortion rate in cattle. In a group of 3,884 cows observed by Gilman (17) over a 7 year period, those free from brucellosis had an abortion rate of approximately 2.3%. In South Dakota at an estimated annual cost of $100 to $120 to maintain a brood cow in the western portion of the state, a 2% abortion rate would result in a loss of 4 to 4.8 million dollars annually. (31) The monetary loss to dairymen in Vermont has been estimated to be roughly equal to the amount they pay annually in property taxes. (4)

In recent years results of a number of surveys have shown infectious bovine rhinotracheitis (IBR) virus to be one of the most commonly diagnosed causes of bovine abortion. (19,31,52) Kirkbride et al. (31) found IBR infection to be the cause of nearly 16% of 2,544 abortions submitted to the South Dakota Animal Disease Research and Diagnostic Laboratory over a 4 year period. Infectious bovine rhinotracheitis virus was first described as the cause of an upper respiratory tract infection

of cattle in Colorado feed lots in 1950 and large commercial dairies in California in 1953. (38) Results of subsequent studies have also shown this virus to be the cause of infectious pustular vulvovaginitis (IPV). (25) Between 1949 and 1959, IBR-IPV infections were not associated with abortion, (52) because abortion was not a recognized clinical manifestation of the infection. Infectious bovine rhinotracheitis-infectious pustular vulvovaginitis abortions usually occur in apparently recovered animals at some time following the cessation of overt clinical symptoms of the infection or, in many cases, no clinical signs other than abortion occur. Therefore abortion was not correlated with the disease. (36) Exposure to a virulent field strain of the virus and parenteral inoculation of a live virus vaccine have been incriminated as sources of IBR infection. (28,36,43) After exposure to the field strain of IBR, virus replication occurs in the mucosa of the upper respiratory tract, and the virus is spread hematogenously to the fetus via the placenta. (52) The fetus, with the possible exception of the immediate prenatal period, is highly susceptible to the IBR virus. Infection brings about a peracute infection that is almost always fatal, and the fetus is expelled 4 to 7 days after death. (27) The aborted fetus and especially its membranes are severely autolyzed and edematous. At the time of fetal death the log $TCID_{50}$ of virus per gram of tissue ranges from 3.5 to 6.0. In the period between fetal death and expulsion from the uterus, the level of virus in the fetal tissue decreases and in some cases can not be isolated from the aborted fetus. (27) Infectious bovine rhinotracheitis virus antibody has rarely been detected in the diseased fetus, probably

because of the rapid death of the fetus after infection. (29) If virus isolation is attempted, the cotyledonary villi of the placenta should be among the tissues examined, as the virus seems to persist longer after fetal death in these tissues than in the fetus. (29,30,52) In the bovine fetus, IBR produces lesions that are characterized by wide-spread necrosis and a limited inflammatory reaction. Most of the necrosis is focal in nature and can occur in almost any tissue of the fetus with the liver being affected in every case. Massive necrosis may destroy almost all of the kidney cortex. (27) The fluorescent antibody test (FAT) is a successful and specific means of diagnosing IBR abortion. The infected kidney and adrenal gland fluoresce most consistently and intensely and are thus the preferred tissues for the FAT examination. (30)

Another virus that has been associated with bovine abortion is the bovine viral diarrhea (BVD)-mucosal disease (MD) virus. In the first description of BVD-MD associated abortion, 20 abortions occurred among 95 cows (of which an unknown number were pregnant) and 32 pregnant heifers. (41) Abortions occurred 10 days to 3 months after the onset of the disease. In contrast to the literature of 10 to 15 years ago, present information indicates that the rate of BVD abortion is not high. (26) Kahrs (21) estimates that in the northeastern United States, 1 per 1,000 bovine pregnancies is aborted by BVD. These losses are not distributed evenly in the population, but are clustered in time and space.

Bovine viral diarrhea infection of susceptible pregnant cattle can be followed by normal calving, abortion, stillbirth, mummified fetuses, or

the birth of calves with various congenital defects. (8,21) The effect the virus has on the fetus is dependent on the age of the fetus at the time of infection. When infection occurs during the first trimester, the fetus undergoes severe disease which generally results in death. Most fetuses affected in this way are aborted, but at this stage it is easy for an abortion to occur and not be observed. (8,26) If infected during the second trimester, the fetus usually survives the disease, but may develop teratologic defects of the brain, skin, or bronchioles due to necrosis of the developing tissues. The most commonly observed defect is cerebellar hypoplasia which may be associated with defects of the retina. (7,8, 23,24,26) Fetuses that survive the infection have high BVD antibody levels at birth before the ingestion of colostrum. (26) Fetuses infected during the third trimester undergo a mild disease from which they usually recover completely. These fetuses also have high BVD antibody level at birth, before ingestion of colostrum. (26) The prenatal increase in resistance to the disease can be expected as protective mechanisms, including the immune system, develop and begin to function. (8)

Several methods are used in the diagnosis of BVD abortion. It is important to: 1. establish through herd history and serologic means that BVD infection was active in the herd just prior or during the occurrence of the abortions 2. demonstrate the presence of active, specific neutralizing antibodies to BVD in the serum from the aborted fetus 3. demonstrate fetal lesions and/or the tetratologic defects compatible with in utero BVD infection. 4. If possible, isolate the BVD virus from the aborted fetus. Virus isolation is difficult for a number of reasons.

Abortion follows fetal death by several days during which time autolysis and the disappearance of the virus from the fetus occurs. (8) The fetus is capable of producing specific BVD neutralizing antibodies that are significant in the elimination of the virus from the fetus. (5,22) Some BVD virus strains found in the fetus are noncytopathic, and are thus demonstrable only by FAT, and indirect techniques such as inoculation into susceptible calves, or induction of cellular resistance to cytopathic strains. (22)

Parainfluenza-3 (PI_3) virus was first reported isolated from an aborted calf by Sattar et al. in Ohio. (48) Sugimura et al. (53) have also reported the isolation of PI_3 virus from an aborted fetus. In utero inoculation of PI_3 virus has been shown to cause fetal death. (55,48) The prevalance of bovine abortion due to PI_3 is not known because recovery of the virus from aborted fetuses appears difficult. (55) However, Dunne et al. (13) found PI_3 hemagglutination inhibition (HI) antibodies in 53% of the aborted fetuses he examined. In 24% of the abortions, PI_3 HI antibody was the only antibody detected.

To a lesser extent many other viruses have been isolated from aborted calves. Dierks et al. (11) reported on the isolation of bovine adenovirus subgroup 2 from 2 aborted fetuses, a stillborn calf and new born calves with weak-calf syndrome. Swanepoel and Blackburn (54) in Rhodesia have isolated an arthopod-borne virus with the characteristics of a member of the Palyam serogroup of the orbiviruses. This virus, which was given the name Nyabira virus, was found in 7 of 574 aborted bovine fetuses. Kurogi et al. (33) have reported the isolation of Akabane virus from aborted calves. The virus has been shown to be

associated with abortion, stillbirths, and the arthrogryposis-hydranencephaly (A-H) syndrome of calves (20,33). Luedke et al. (34) have shown bluetongue (BT) virus to be abortogenic. Two of 10 pregnant heifers aborted after being exposed to BT virus through the bites of an infected biologic vector, Culicoides variipennis, at 60 and 120 days gestation. Of the remaining heifers, one gave birth to a stillborn calf, and the other 7 gave birth to calves with various degrees of gross dysfunctions and anomalies. Results of other studies have also shown that arthopod born viruses may be involved with bovine abortion. (37,58).

A bovine parvovirus has been isolated from an aborted fetus by Sugimura et al. (53) Crandell et al. (10) have isolated a herpesvirus from the lung of an aborted bovine fetus. This herpesvirus has been shown by serum neutralization tests to be distinct from IBR, DN-599, Movar 33/63, bovine mammallitis, malignant catarrhal fever, feline viral rhinotracheitis, equine herpesvirus I, and pseudorabies. These reports and others show that a wide variety of viral agents may be involved with bovine abortion.

In a survey of the causes of abortions and stillbirths in the Northern Plains States, nonviral causes of abortions included the following: Mycotic abortion 3.5%, vibriosis 3.0%, miscellaneous bacterial infections, dystocias, and anomolies 7.4%. (31) A similar survey in the northeastern states found Aspergillus sp. streptococci, leptospira, Vibrio sp. and Corynebacterium sp. to be the most common nonviral causative agents of bovine abortion. (19) Surveys in Vermont and Canada gave similar results. (4,40)

There have been several reports of the isolation of mycoplasma from

aborted fetuses and the bovine genital tract. (14,31,42,50,56) Gourlay (18) states that the role of mycoplasma in bovine genital disease is uncertain, but he does consider them potential agents of reproductive disease. Early attempts to infect pregnant cows with mycoplasma proved ineffective (50), but Stalheim and Procter (51), in 1976, were able to induce abortion in 2 pregnant cows 11 and 18 days after Mycoplasma agalactiae subsp. bovis was inoculated into the amniotic fluids. Stalheim and Procter, however, state that the significance of mycoplasma in bovine abortion remains to be determined.

As documented earlier, these aforementioned viral and bacterial agents represent the cause of only approximately 30% of the abortions. These figures indicate that conventional microbiologic, histologic, and serologic examinations do not produce the desired percentage of diagnoses of bovine abortion. Because these techniques are most effective in detecting abortions caused by infectious agents, they would not be particularly effective in over-all diagnosis if a large portion of bovine abortions were caused by non-infectious agents. There are indications, however, that infection is associated with a large number of abortions. Histologic examination of fetal tissues reveals lesions suggestive of infection in a large percentage of abortions. Miller and Quinn (39) found lesions in 48 of 50 aborted fetuses, while in 50 non-aborted fetuses collected at an abattoir, only 12 had lesions. In New Zealand, lesions were found in 46 of 103 calves aborted because of unidentified causes. (32) In another 31 aborted fetuses, possible lesions were observed but autolytic changes made interpretation difficult. At the South Dakota Animal Disease Research and Diagnostic Laboratory the examinations of

506 fetuses for which the cause of abortion could not be determined revealed lesions in 330 of these fetuses. (12)

Results of serologic tests also seem to indicate infection (or at least antigenic stimulation of the fetus) is frequently associated with bovine abortion. In New Zealand, immunoglobulin (Ig) in the serum of 164 aborted and 59 abattoir bovine fetuses was measured by radial immunodiffusion (RID). Abnormally high Ig content was detected in the serum of 81.1% of the aborted fetuses and 10.2% of the abattoir fetuses. (32) Miller and Quinne, (39) using immuno-electrophoresis, detected abnormally high levels of Ig in 44% of 50 aborted fetuses, whereas only 12% of 50 abattoir fetuses had elevated Ig levels. Observations at the South Dakota Animal Disease Research and Diagnotic Laboratory show elevated Ig levels in 30.6% of 516 aborted calves and 11.8% of 118 abattoir fetuses. (12) If it is assumed that infection is the most common cause of prenatal antigenic stimulation, then these data indicate that infection occurs more often in aborted calves than in calves taken at slaughter.

Since the development of the negative stain spray technique by Brenner and Horne (6) in 1959, much work has been done with negative stain electron microscopy in viral research. Sprawbrow and Francis (49) demonstrated that viral particles could be seen rapidly and routinely in infected cell cultures lysed with distilled water. With these techniques it was possible to determine to which group newly isolated viruses belonged within hours of the first recognition of cytopathic effect (CPE). Gibbs and Johnson (16) used negative stain electron microscopy for the direct demonstration of virus in lesions of bovine teats. With negative

stain the electron microscope can be used to determine viral structure, and this can have practical application in routine veterinary diagnosis. (35) Herpesvirus, bovine mammillitis virus, pseudocowpox virus, and cowpox virus were observed in scrapings from bovine teat lesions. (16,35) Papilloma virus has been seen in samples from the "fleshy" areas of warts. (35) Reovirus, rotavirus, and coronavirus-like particles have been observed in fecal material from neonatal calves, (59) pigs, (3,47) and humans. (3) Reovirus-like agents have also been observed in the feces from a neonatal pronghorn antelope. (44) At the south Dakota Animal Disease Research and Diagnostic Laboratory, negative stain EM is used daily to determine the presence of virus in a wide variety of specimens. Fecal material, tissue, and cell culture extracts have been examined to demonstrate the presence of a variety of viruses.

Immuno-electron microscopy (IEM) was first used by Anderson and Stanly (1) in 1941, to study the reaction between tobacco mosaic virus and its specific antibody. Immuno-electron microscopy has been used to detect and/or serotype a number of viruses including rotavirus, transmissible gastroenteritis virus, (47) adenovirus, (57) picornovirus, (9), and the corona-like virus characteristic of turkey bluecomb disease. (45)

Materials and Methods

Source of Specimens

Bovine fetal tissues or entire fetuses were received at the South Dakota Animal Disease Research and Diagnostic Laboratory from veterinary practitioners in South Dakota, western Minnesota, northern Nebraska, and southern North Dakota. Fetal tissues submitted usually included lung, liver, spleen, kidney, and stomach contents. Placental material was submitted when it was available. When the entire fetus was submitted, a complete necropsy was performed at the laboratory.

Collection and Examination of Specimens

Tissues submitted by veterinarians as well as tissues collected at necropsy were placed in sterile plastic bags and examined for bacteria, viruses, and fungi the same day. Lung and liver for electron microscopic examination were placed in a separate sterile plastic bag and examined that day or held frozen at -20 C. Fetal serum or body fluid for radial-immunodiffusion (RID) was also obtained, centrifuged 15 min at 2,000 xg to remove solid matter, and stored at -20 C until tested. Tissues for histologic examination were fixed in buffered 10% formalin. Bacteriologic, histologic, mycotic, and virologic examinations were done according to methods described by Kirkbride et al. (31) Methods of Fahey and McKelvey (15) were used to measure the levels of serum gamma globulin by RID.

Electron Microscopy (EM)

Grid preparation: An evaporating dish 20 cm x 7 cm was placed in a dust and draft free environment and filled to overflowing with distilled water. A glass rod was used to skim any dust from the surface of the

of the water. Using a pasteur pipette, 4 drops of 1% nitrocellulose (collodium) in amyl acetate[a] were dropped in rapid succession onto the water to form a thin even film. Copper grids (300 mesh, rhodium coated on one side)[b] were floated, rhodium side down on the collodium film. The grids were arranged to form a 2.5 cm x 6 cm raft in the center of the collodium film. The array of grids and collodium were then picked up with a strip of 2.5 cm (1 inch) wide standard masking tape, using a rolling motion to attach, by surface tension, the non-adhesive side of the tape to the upper surface of the grid array. The adhesive side of the tape was attached to a glass microscope slide, then set aside to dry in a dust free environment. The dry collodium coated grids were then coated with a thin carbon film using a vacuum evaporator[c].

Tissue preparation: Approximately 0.5g each of lung and liver tissue were homogenized together in 7 ml Hank's balanced salt solution (HBSS)[d] using a Ten Broek grinder. The suspension was centrifuged 15 min at 1,085 xg at 4 C in a refrigerated centrifuge[e]. The pellet was discarded. The resulting supernatant fluid was centrifuged 30 min at 39,000 xg, at 4 C, and the supernatant discarded. The pellet was refrigerated and examined within 8 h.

Negative stain EM: The pellet was resuspended in 0.1-0.5 ml distilled

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a,b Ernest F. Fullam Inc., Schenectady, New York

c Illini Consultants, no longer in business

d Grand Island Biologic Co., Grand Island, New York

e Beckman J-21-B, Beckman Instruments, Inc. Palo Alto, California

water, the volume depending on the size of the pellet. On a spot plate, one drop of this suspension was added to a solution of 20 drops of distilled water, 2 drops of 4% phosphotungstic acid (PTA)[f] pH 6.4, and one drop of 0.1% bovine serum albumin (BSA)[g]. The solution was mixed, and using a glass nebulizer[h], was sprayed onto a carbon and collodium coated grid. The grid was then examined immediately using an electron microscope[i] at a magnification of 20,000 X. If no virus-like particles were observed after scanning the grid for 10 min, the sample was considered free of virus.

Immuno-EM: Two drops of the same water-virus preparation used for negative stain EM were mixed with three drops of a known antiserum. This mixture was incubated overnight at 4 C. In order to retrieve the virus-antibody complex, the mixture was centrifuged 60 min at 80,000 xg, at 4 C in a preparative ultracentrifuge[j]. The resultant pellet was resuspended in a mixture of 1 drop 4% PTA, 1 drop 0.1% BSA, and 10 drops distilled water. The specimen was then sprayed onto a grid and examined by the same method used for the negative stained samples. The grid was examined to determine if antigen-antibody reaction had occured. If antibody could

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f Mallinckrodt Chemical Works, St. Louis, Missouri

g Miles Laboratory, Elkhart, Indiana

h Pelco Corp., Tustin, California

i Hitachi HU-12, Hitachi Ltd. Tokyo, Japan.

j IEC model B-60. International Equipment Co., Needham, Massachusetts

be seen covering the virus, or if aggregates of viruses connected by antibody could be seen, the antiserum was considered specific for the virus.

Virus Isolation

Media: The growth medium for all cell lines was Eagle's Minimum Essential Medium (MEM-10%) in Earle's balanced salts with nonessential amino acids[k], supplemented with 10% fetal calf serum, o.1% pyruvic acid, 0.03% L-glutamine, 0.5% lactalbumin hydrolysate, and the following antibiotics: 100 units/ml penicillin G, 100 ug/ml streptomycin, and 100 ug/ml kanamycin. A similar medium (MEM-2%) in which the final fetal calf serum concentration was 2% was used to maintain cell cultures during virus isolation attempts. Maintenance medium (MEM-t) for the 1721 virus-like particle (see results) virus isolation attempts was Eagle's MEM with 5 ug/ml trypsin, supplemented with 0.5% BSA, and 0.03% L-glutamine.

Medium for plaque assay consisted of double strength Eagle's MEM supplemented with 0.01% pyruvic acid. This was mixed with an equal volume of melted 1.4% Ion agar[1] containing 40 ug/ml DEAE dextran. The Eagle's medium and the Ion agar were equilibrated to 45 C before being mixed, and the mixture was held at this temperature until it was added to the cell monolayers.

Cell culture: Continuous cell lines used for virus isolation

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k Grand Island Biologic, Grand Island, New York

1 Colab Laboratory Inc., Science Park, Glenwood, Illinois

included: African Green Monkey kidney (BSC-1)[m], bovine turbinate[n], Maden Darby bovine kidney[n], and pig kidney (PK-15)[n].

Cultures of secondary bovine intestinal cells were established using intestinal tissue collected from fetuses obtained at slaughter. Approximately 1 g of intestinal tissue was finely minced, then stirred in 25 ml of Eagle's MEM to remove microvilli and mucus. After 60 min, the suspension was centrifuged 10 min at 800 xg, at 24 C, the pellet was resuspended in 25 ml of 0.25% trypsin, and the mixture was stirred with a magnetic stirrer 20 min at 37 C. The supernatant containing the individual cells was decanted and centrifuged 10 min at 800 xg, at 24 C. The cell pellet was washed twice with 10 ml HBSS. Growth was initiated by resuspending the packed cells 1:50 in Eagle's MEM-10% and seeding 10 ml of this suspension into Corning 25 cm^2 tissue culture flasks[o].

Virus isolation: Approximately 0.5 g of the tissue to be examined was homogenized in 7 ml HBSS using a Ten Broek grinder. The homogenate was centrifuged 20 min at 3,000 xg at 4 C. One ml of the resulting supernatant was inoculated onto fresh monolayers of each of the following cell lines growing in Corning 25 cm^2 tissue culture flasks: BSC-1, MDBK, PK-15, and bovine intestinal cells. After a 1 h adsorbtion period the inoculum was replaced with 10 ml Eagle's MEM-2%. In one uninoculated flask of each cell line in the same passage, the growth medium was replaced with MEM-2%. These were used as cell controls. Cell cultures

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[n] National Animal Disease Center, Ames, Iowa

[o] Scientific Products, Minneapolis, Minnesota

were incubated at 37 C and examined daily for cytopathic effect (CPE). If no CPE was observed after 10 days, the cultures were frozen, thawed, and 1 ml of the culture cell suspension was inoculated onto a fresh monolayer of cells. Cultures were considered free of virus if after 4 blind passages no CPE was observed and no virus could be demonstrated by EM examination of negative stain preparations.

Tissues thought to contain the 1721 particle were submitted to the regular culture procedures, and in addition, were cultured by the method described by Babiuk et al. (2). Tissue homogenates were prepared as previously described. Each homogenate was treated with an equal volume of Eagle's MEM containing 500 ug/ml trypsin. The mixture was incubated 15 min, at 37 C, diluted 1:20 with Eagle's MEM, and 1 ml was added to a fresh monolayer of each of the aforementioned cells growing in 25 cm^2 tissue cuture flasks. After a 2 h adsorption period, the inoculum was decanted and replaced with 10 ml MEM-t. To provide cell controls, in one uninoculated flask of each cell line of the same passage the growth medium was replaced with MEM-t. Cultures were incubated at 37 C and examined daily for CPE. After 10 days or as soon as the cells had undergone extensive cytopathic degeneration, the supernatant fluid was removed and replaced with 5 ml 0.025% trypsin, which was poured off after 30 sec. The culture flask was incubated approximatlely 10 min, at 37 C until the remaining cells floated free. Two ml Eagle's MEM containing 10 ug/ml trypsin was then added. The cells were frozen, thawed, then incubated 10 min, at 37 C, after which 2 ml Eagle's MEM was added to give a total volume of 4 ml. After the suspension was mixed, 1 ml was inoculated onto

a fresh monolayer of cells, and after 2 h incubation, the inoculum was replaced with 10 ml Eagle's MEM-t. If after 4 passages no CPE was observed and virus could not be seen using negative stain EM, the sample was considerd free of virus.

Plaque Assay

Plaque assay was attempted to enumerate the 1721 virus isolated from tissue culture, and as a means of evaluating the effect of lipid solvents and specific antisera on the virus. Bovine turbinate and BSC-1 cells were incubated in Falcon #3008 24-well tissue culture plates[p] until a complete monolayer was formed on the bottom of each well. The growth medium was removed and an inoculum of 0.1 ml of each 10 fold virus dilution in HBSS was placed into duplicate wells. Two wells of each cell type were not inoculated with virus and served as cell controls. After adsorbing for 1 h, the inoculum was removed, and 1 ml of the agar-MEM-2x overlay was added to each well and allowed to solidify at room temperature. The culture plates were inverted and incubated at 37 C. After 2-3 days, or when CPE was observed, the agar was removed and 0.2 ml of 10% crystal violet in phosphate buffered 10% formalin (pH 7) was added to each well to fix and stain the cells. After 3-5 min the stain was poured off and the plaques counted to determine the virus titer as plaque forming units/ml.

Lipid Solvent Sensitivity

Chloroform was used to determine if the 1721 virus was sensitive

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[p] Falcon Plastics, Oxnard, California

to lipid solvents. One ml of the tissue culture isolate of the 1721 virus was added to 1 ml chloroform and the mixture was shaken 6 times (30 sec/shake) at even intervals over a 30 min period. The emulsion was centrifuged 10 min at 2,000 xg, at 4 C, to separate the layers. The aqueous layer, containing the virus was decanted. Serial 10-fold dilutions were made of the treated and untreated virus suspensions. Using the plaque assay method decribed earlier, both sets of dilutions were then titrated to determine if chloroform treatment reduced the number of PFU's as compared to the untreated culture.

Serum Neutralization

The 1721 virus was reacted with antiserum against the La Crosse strain of California encephalitis virus[q] to determine if the serum had any neutralizing effect on the virus. An aliquot of the antiserum to be tested was diluted 1:5. Serial 10-fold dilutions to 10^{-10} were made of the tissue culture isolate of the 1721 virus, and 0.2 ml of each dilution was placed into separate test tubes. To each test tube, 0.4 ml of the dilute antiserum was added. Ten-fold dilutions of untreated virus as well as the virus-antiserum mixtures were incubated 1 h at 37 C, then plaqued to determine if the serum reduced the virus titer as compared to the untreated virus dilutions.

Mouse Inoculation

Tissue homogenates were prepared as for virus isolation and centrifuged 20 min, at 3,000 xg, at 4 C. Using a sterile tuberculin syringe

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[q] Center for Disease Control, Atlanta, Georgia

with a 26 gage needle, 0.01 ml of the resultant supernatant was inocultated intracranially into 7 24 h old suckling mice. The mice were observed daily for loss of appetite, paralysis, or death. If they did not die within 2 weeks after inoculation, they were killed at this time. In either instance, their brains were removed, pooled, homogenized in 7 ml HBSS, centrifuged 20 min at 3,000 xg, at 4 C, and 0.01 ml of the supernatent was inoculated intracranially into 7 24 h old suckling mice. If after 3 passages through mice no effects were observed, and negative stain EM examinations of the pooled mouse brains did not reveal any virus, the sample was considered free of virus.

Egg Inoculation

The material prepared for tissue culture inoculation was also used to inoculate 7-day-old embryonated chicken eggs. An aliquot (0.1 ml) of the sample was injected into the allantoic cavity of each of 4 embryos and into the yolk sac of each of 4 other embryos. The embryonated eggs were candled daily, and after 10 days or embryonic death without bacterial contamination, whichever came first, the allantoic fluid or the yolk sac was aseptically removed. The respective samples were pooled, homogenized in HBSS, centrifuged 20 min at 3,000 xg, at 4 C, and 0.1 ml of the supernatant was inoculated by the same route into 7-day-old chick embryos. If the inoculum did not kill the chick embryos within 10 days on the 3rd passage, and EM examination did not reveal any virus, the sample was considered free of virus.

An aliquot (0.1 ml) of the material prepared for tissue culture inoculation was also inoculated intravenously into 6 12 to 13-day-old chick embryos. Ten days after inoculation or when nonbacterial embryonic death

occured prior to then, the embryo was removed, homogenized in HBSS, centrifuged 20 min at 3,000 xg, at 4 C, and 0.1 ml inoculated into embryonated chicks via the same route. Again, if fetal death did not occur within 3 passages, and EM observations were negative, the sample was considered free of virus.

Results

Results of the negative stain EM examinations of the lung and liver tissues from 500 aborted bovine fetuses are given in Table 1.

In 30 cases in which herpesvirus was observed by EM, the virus was confirmed as IBR by FAT, and lesions typical of IBR were observed in the tissues from the aborted fetus. (Figures 1, 2, Table 2) In the other 5 aborted fetuses the identity of the particles with herpesvirus characteristics was not established; however, one of these cases was diagnosed as enzootic abortion, and one had placentitis. One of the fetuses containing these particles had microabscesses of the liver, but no lesions were observed in the remaining 2 fetuses. Viruses were not isolated from these 5 abortions using routine cell culture systems.

A dense fibrous matrix (designated 1721), 50-70 nm in diameter, resembling unencapsulated viral nucleic acid, often closely associated with a cellular membrane (Figures 3, 4) was seen in the tissues of 8 fetuses. The results of laboratory examinations of these 8 fetuses are summarized in Table 3. Microscopic lesions were present in the tissues of 7 of these fetuses. Lesions which occurred most consistently were interstitial pneumonia, thrombosis, and forms of hepatitis. Serum Ig concentrations were measured in the body fluids from 4 of the 8 fetuses. None had Ig levels >20 mg/dl. Twenty mg/dl is the maximmum concentration considered normal. (32) Immuno-EM of the 1721 particle using the crude tissue extract was attempted, these results are summerized in Table 4. Some antigen-antibody reaction was observed when the antiserum against La Crosse strain California encephalitis virus was reacted with the 1721

Table 1. Virus-like particles observed by EM examination of negative stained liver and lung tissues from 500 aborted bovine fetuses

Particle[a]	Number of fetuses	% of 500
Herpesvirus	35	7.0
Coronavirus-like	9	1.8
Unidentified (1721)[b]	8	1.6
Influenzavirus-like	1	0.2
Reovirus-like	1	0.2
Phage	2	0.4
Total	56	11.2

a Identified by morphologic features only. True identity was not established.

b These particles all had similar morphologic characteristics.

Table 2. Comparison of the number of cases diagnosed by routine laboratory procedures as IBR abortion with the number of fetuses in whose tissues herpesvirus was seen by EM examination

Year	Number of Cases examined by EM	Number of Cases IBR Positive[a]	Number of Cases herpes Positive by EM	Cases both Herpes EM and IBR positive[b]	% correct EM IBR Diagnosis
1976	65	5	1	1	20%
1977	358	23	17	14	60%
1978	77	15	17	15	100%
Total	500	43	35	30	70%

a By histopathology and FAT

b The identity of the particle was not established in the five cases which were not IBR positive. No lesions were observed in two of these cases; one was diagnosed as enzootic abortion; placentitis was observed in one of the fetuses; and focal hepatitis was observed in the last fetus. All virus isolation attempts on the tissues of these fetuses failed.

Table 3. History and results of laboratory examinations of fetuses in which the 1721 virus particle was found

Case #	Received	Location	Fetal age	RID[a] (mg/dl) IgG	RID[a] (mg/dl) IgM	Histopathology
77-1721	2/22/77	Highmore SD	6 mo	0	0	mild pneumonia, centrilobular necrosis and pericholangitis in the liver
77-2349	2/25/77	Mitchell SD	UK[b]	NA[c]	NA	centilobular necrosis of the liver
77-2467	4/4/77	Sundance WO	UK	NA	NA	no visible lesions
77-2474[d]	2/28/77	Elkton SD	7 mo	10	0	bronchitis and mild pneumonia, mild portal and subcapsular hepatitis, RE hyperplasia of the lymph nodes, edema of the cerebrum
77-3709	3/23/77	Dell Rapids SD	7 mo	12	15	congestion of the brain meningeal hemorrhage
77-4367	4/4/77	Elkton SD	8 mo	0	0	lymph nodes active, RE cells prominent
77-14597	12/13/77	Hoffman MN	7 mo	NA	NA	centrilobular necrosis of the liver, RE hyperplasia and pulmonary edema
77-15123	12/29/77	Presho SD	UK	NA	NA	hepatitis

a Radial immunodiffusion
b Fetal age not known
c Fetal serum or body fluids not available
d Diagnosed as mycotic abortion, Aspergillus fumigatus isolated

Table 4. Results of immuno-electron microscopy of the 1721 particle observed in the tissues of abortion number 77-15123

Antiserum	Reaction
Reo-2[a]	-
Reo-3[b]	-
BVD[c]	-
La Crosse strain Ca. encephalitis[a]	±
Western encephalitis[a]	-
Bunyamwera[a]	-
Blue tongue[c]	-
Epizootic hemorrhagic[c] disease of deer	-

a Center for Disease Control, Atlanta, Georgia
b Grand Island Biologic, Grand Island, New York
c Animal Disease Research and Diagnostic Laboratory, Brookings, South Dakota

virus procured by extract of fetal tissue. No reaction was observed when the particle was reacted with the other antisera listed in Table 4. All virus isolation attempts using routine cell culture, suckling mice, and embryonated chicken eggs failed. Attempts were then made to isolate the agent from the tissues of cases 77-14597 and 77-15123 using the method of Babiuk et al. (2) On the bovine turbinate cell culture of case 77-15123, CPE became evident 72 h post inoculation (PI). The CPE consisted of the formation of giant multinucleated cells (syncytia), some vacuolization, and the rounding of some of the cells. After passage of this culture, CPE occurred 48 h PI and consisted primarily of rounding of the cells and their subsequent release from the monolayer, along with some syncytia formation and vacuolization. Electron microscopic examination of this culture revealed particles approximatly 60 nm in dia. (Figure 5) and an abundance of membrane material. This membrane material did not seem to be closely associated with the virion. Results of IEM examination of this tissue culture isolate are given in Table 5. The virus reacted with the antiserum to the La Crosse strain the California encephalitis virus (Figure 6) Some antigen-antibody reaction was observed with the antisera to St. Louis encephalitis, Western encephalitis, and a California encephalitis virus group. The third passage of this isolate was inoculated into suckling mice, the results are given in Table 6. Electron microscopic examination of an extract of the brain material of the mice inoculated with the tissue culture diluted 1:100 revealed a virus similiar in structure to the tissue culture isolate. Immuno-EM was not attempted due to a shortage of suitable brain material. Attempts were

Table 5. Results of immuno-EM of the bovine turbinate cell culture infected with the 1721 virus from bovine abortion 77-15123

Antiserum	Reaction
Reo-2[a]	-
Reo-3[b]	-
Blue tongue[c]	-
Epizootic hemorrhagic disease of deer[c]	-
La Crosse Strain Ca. encephalitis[a]	+
Western encephalitis[a]	±
Ca. encephalitis (group antiserum)[a]	±
St. Louis encephalitis (TBH-28)[a]	±
Bunyamwera[a]	-
Flanders[a]	-
Turlock Len 788-19	-

a Center for Disease Control, Atlanta, Georgia
b Grand Island Biologic, Grand Island, New York
c Animal Disease Research and Diagnostic Laboratory, Brookings, South Dakota

Table 6. Results of suckling mice inoculation[a] with 1721 virus-bovine turbine isolate 77-15123

Virus Dilution	Time of Death (days)	Mortality Rate
0	12-13	7/7
1:10 (b)	12-13	7/7
1:100	12-13	5/7
Control	-	0/7

a 0.001 ml IC inoculum

made to enumerate viruses in the third passage using the plaque assay procedure; however, plaques were never observed. Similar results were obtained with the lipid solvent sensitivity test and the serum neutralization experiment. Aliquots of this third passage isolate were frozen for future reference. This isolate was also sent to the Arbovirus reference branch of the center for Disease Control, Fort Collins, Colorado. At the time of this writing the isolate has not been identified.

Particles with morphologic characteristics of coronavirus were seen in tissues of 9 fetuses. (Figure 7) Histologic examinations (Table 7) revealed lesions in the tissues of 7 of these fetuses. The lesions occurring most consistently were intersitial pneumonia, periportal hepatitis, and thrombosis. One of the 9 fetuses had mycotic placentitis, and another had hydrocephalus, arthrogryposis, and muscle dysplasia. Serum Ig concentrations were measured in the body fluids from 6 of the 9 fetuses. One fetus had 24 mg/dl IgG and 60 mg/dl IgM. Immunoglobulin levels in the other fetuses were <20 mg/dl. Attempts to isolate these viruses by routine cell culture and inoculation of embryonating chicken eggs produced no results.

Tissues of one fetus contained particles morphologically resembling influenza virus. However, there were no lesions, and there was no measurable Ig. All attempts to isolate this virus using routine cell culture systems failed.

Particles morphologically similar to reovirus were observed in tissues of one fetus. The fetus had purulent bronchopneumonia, focal necrosis and granulomas of the liver, focal necrosis of the spleen, and granulomas

Table 7. History and results of laboratory examinations of fetuses in which the corona-like virus particle was found

Case #	Received	Location	Fetal age	RID[a] (mg/dl) IgG	RID[a] (mg/dl) IgM	Histopathology
77-820	1/27/77	Bath SD	UK[b]	24	60	vasculitis, pneumonia, nephritis, placentitis, RE hyperplasia of the lymph nodes and liver, necrosis of the thymus
77-1438	2/10/77	Lemmon SD	UK	NA[c]	NA	no visible lesions
77-1721[d]						
77-2006	2/23/77	Watertown SD	8 mo	0	<10	centrilobular hepatic degeneration, focal renal oxalosis, intersitial pneumonia, vascular congestion, nonsuppurative meningitis, placentitis
77-2641	3/9/77	Hazel SD	UK	15	15	portal hepatitis, RE hyperplasia, throbosis of the brain
77-2857	3/14/77	Java SD	UK	NA	NA	lymphoid cuffing of the bronchioles of the lungs
77-3343	3/22/77	Tolstoy SD	UK	NA	NA	edema of the lung
77-3440[e]	3/24/77	Meadow SD	term	10	0	torticollis, arthrogryposis, hydrocephaus
77-3466[f]	3/24/77	Montrose SD	8-9 mo	0	0	pneumonia, hepatitis

a Radial immunodiffusion
b Fetal age not known
c Fetal serum or body fluids not available
d See case # 77-1721 in table 3
e Diagnosed as anomaly probably due to hereditary problems
f Corynebacterium pyogenes isolated, probable cause of abortion

Table 8. History and results of laboratory examinations of fetuses in which miscellaneous observations were made by EM

Case #	EM Observations	Fetal Age	RID[a] (mg/dl) IgG	RID[a] (mg/dl) IgM	Histopathology
77-3164	Influenza-like	8 mo	0	10	No significant lesions
77-4163	Reo-like	Term	0	0	Severe pneumonia, focal necrosis of the liver, RE hyperplasia of the lymph nodes, necrosis of the skelatal muscle fibers.
77-1148[b]	Bacteriophage	6 mo	0	15	Bronchopneumonia
77-1533[c]	Bacteriophage	U.K.[d]	N.A.[e]	N.A.	Calcification of the placenta

a Radial immunodiffusion
b *Campylobacter fetus* variety *venerealis* isolated from the fetus
c *Escherichia coli* isolated in almost pure culture from the fetus
d Fetal age not known
e Fetal serum or body fluids not available

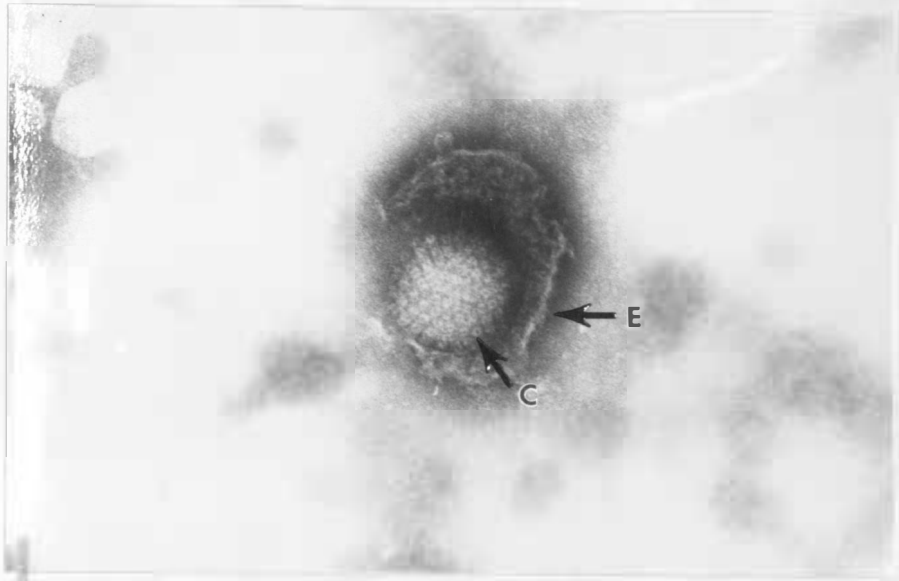
of the renal pelvis. There was no measurable Ig in the body fluids collected from this fetus. Virus was not isolated by routine cell culture systems.

Campylobacter fetus was isolated from one of the two fetuses whose tissues contained bacteriophage, and Escherichia coli was isolated in nearly pure culture from tissues of the other.

Structures resembling mycoplasma (Figure 8) were noted in tissues of approximately 8% of the fetuses examined. Attempts were made to isolate mycoplasma from these tissues without success.

Figure 1. Herpesvirus observed in bovine abortion #78-51. The outer envelope, (E) and the hollow capsomeres (C) are visible. The viral capsid is 108 nm in dia. X 147,000.

Figure 2. Herpesvirus without an envelope observed in bovine abortion #78-51. Hollow capsomeres are visible. (arrow) The viral capsid is 100 nm in dia. X 180,000.


E
C

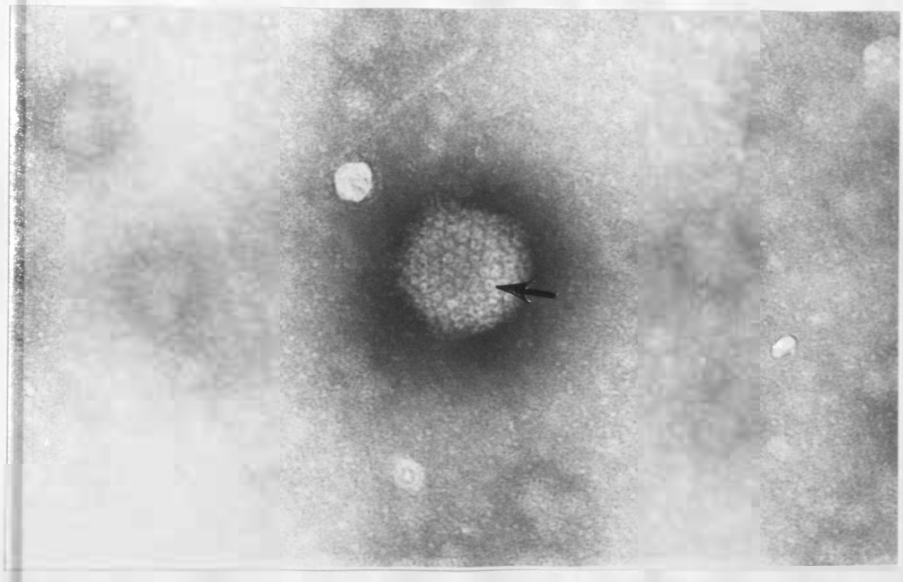
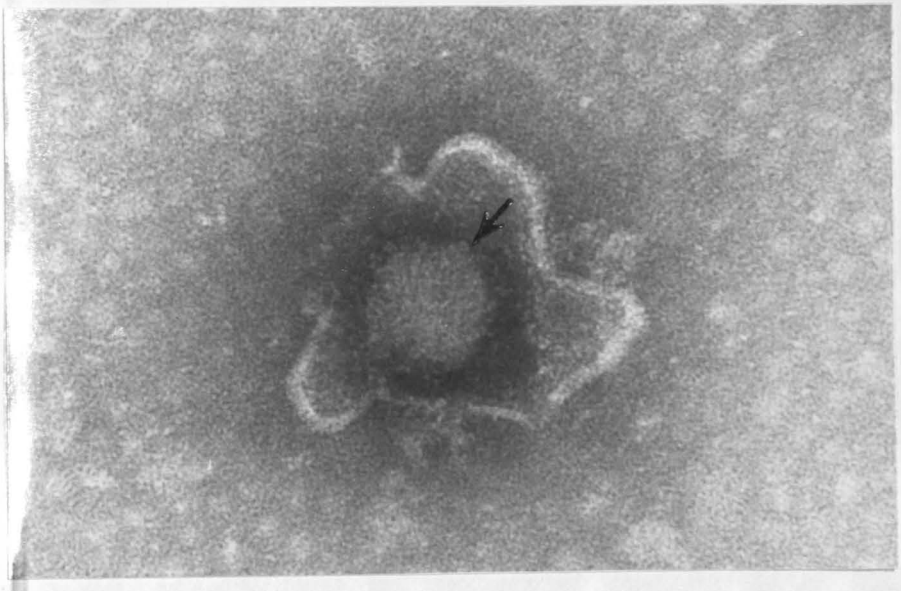


Figure 3. 1721 particle and associated membrane observed in bovine abortion #77-1721. Some capsomere-like structures are visible. (arrow) The capsid is 63 nm in dia. X 287,000.

Figure 4. Multiple 1721 particles associated with a single membrane. The average size of the 4 larger particles is 63 nm. (bovine abortion #77-1721) X 207,000.



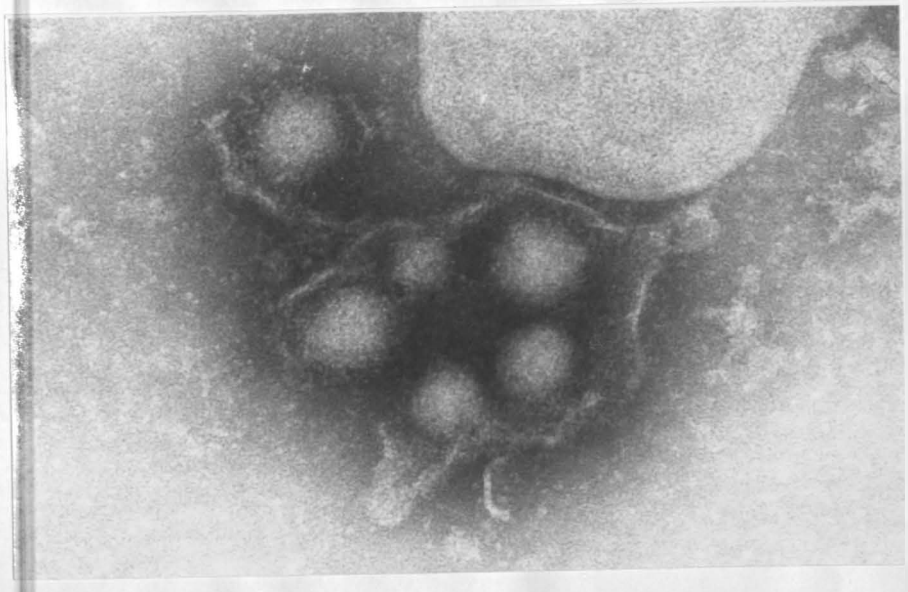
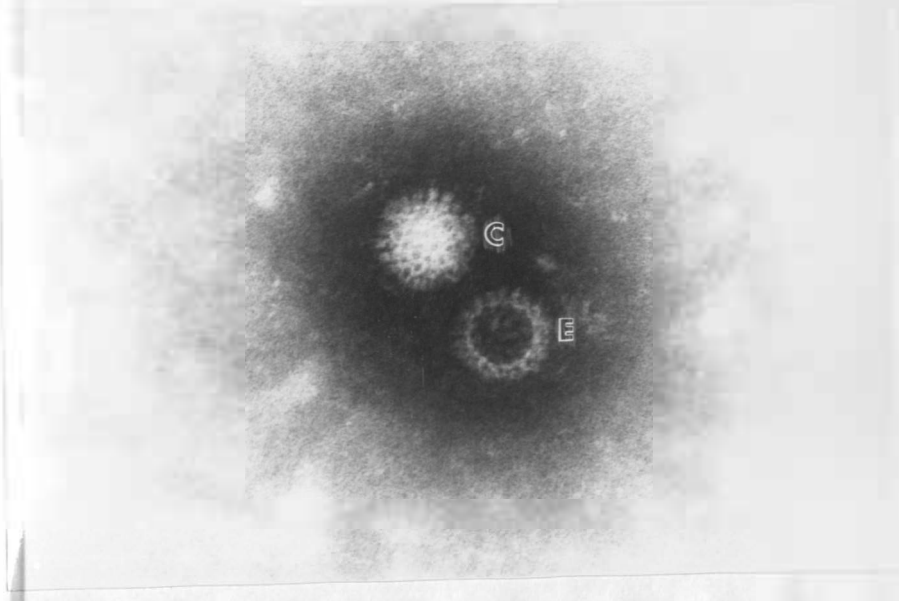


Figure 5. The 1721 virus isolated in bovine turbinate cells from bovine abortion 77-15123. A complete (C) and an empty (E) particle are visible. The particle size is 63 nm in dia. X 190,000.

Figure 6. The 1721 virus that has been reacted with antiserum against the La Crosse strain of the California encphalitis virus. Antibody attachment to the virus is visible. (arrow) X 90,000


C
E

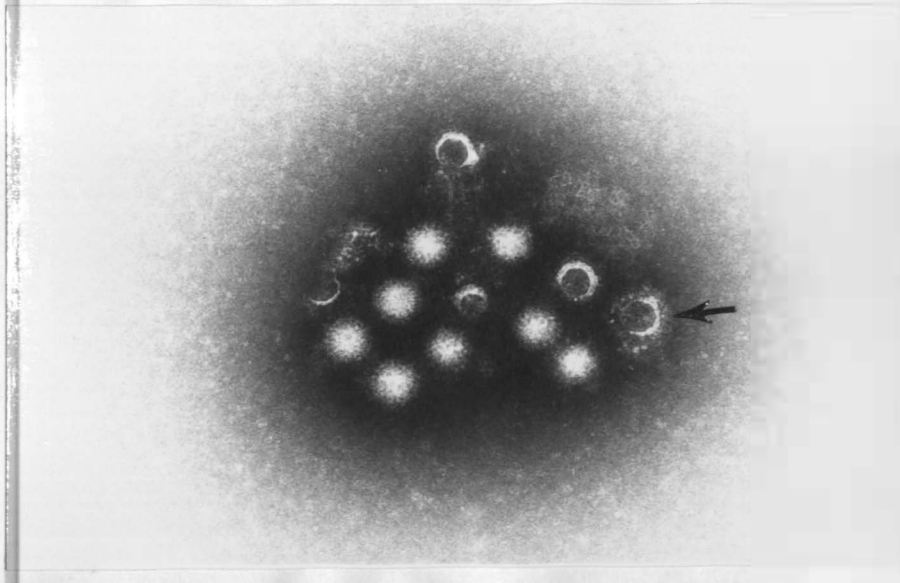
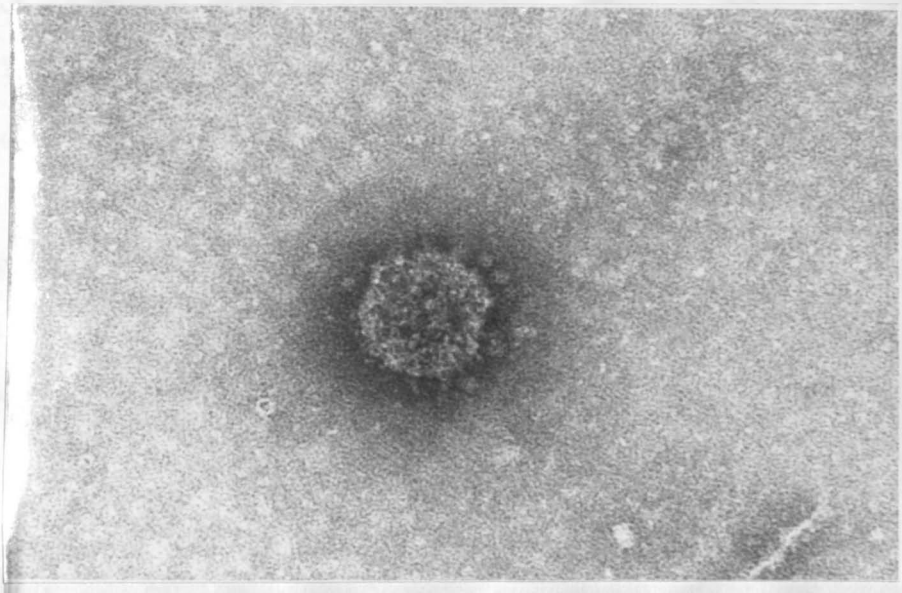


Figure 7. Coronavirus-like object, 68 nm in dia with 12 nm projections, observed in bovine abortion # 77-3343. X 234,000.

Figure 8. Mycoplasma-like objects, probably artifacts of tissue decomposition, observed in bovine abortion # 77-3875. X 112,000.



Discussion

Evidence has accumulated that conventional techniques have failed to identify the infectious agent in a significant percentage of bovine abortions. Therefore, this survey was initiated using the electron microscope. The electron microscopic examination of negatively stained specimens has been established as a useful tool in the diagnosis of viral infections. (16,35,49) The procedure has certain advantages over other techniques; 1. the negative stain procedure is rapid, and the results can be obtained in a few hours, 2. the virus need not be viable to be observed, and 3. the presence of viruses that are not readily grown in cell culture can be determined. However, the procedure has inherent disadvantages: 1. a relatively large concentration of virus particles, approximately 10^5 particles per ml, must be present before a virus can be readily observed by direct EM observation[a], 2. as with other virus identification techniques, merely finding a virus in the tissues does not necessarily establish its significance as an agent of disease, and 3. the size and morphologic structure of the virus can be determined by negative stain EM, but unequivocal identification of the virus requires more information.

Early in this survey, the observation of a herpesvirus in the tissues of a fetus aborted due to IBR infection demonstrated that the technique was at least partially successful. Herpesvirus was seen by EM in the tissues from 20% of the proven cases of IBR abortion in 1976 and in 100%

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a A.E. Ritchie, National Animal Disease Center, Ames, Iowa, personal communication

of the IBR abortions in 1978. (Table 2) The most likely explanation for this increase in success is that experience was gained in the technique and in recognition of herpesvirus. The accuracy of FAT in conjunction with histopathology in diagnosing IBR abortion makes routine EM examination of fetal tissues for IBR unnecessary.

Bovine viral diarrhea virus has been associated with bovine abortion; however, in 500 aborted calves examined by EM, no evidence of BVD virus was found. Because of its pleomorphism, BVD virus is indistinguishable from cell debris. Ritchie and Fernelius (46) reported that for the 3 distinct types of particles found in all strains of BVD virus they examined, the size ranged from 15 to 100 nm.

Coronaviruses are difficult to distinguish from cell fragments in negativly stained preparations. (45) Isolation of coronavirus using routine cell culture systems is difficult, therefore, the identity of the corona-like particle was not definitly established. Similar problems were encountered with the influenza and reo-like particles.

Particles resembling mycoplasma were observed in tissues from approximately 8% of the aborted fetuses examined. Since none of these agents grew when cultured, these particles may have been non-viable or fastidious strains of mycoplasma, or artifacts of tissue decompostion.

The virus designated 1721 has the size and structure of viruses of either the Toga or Reoviridea group, but at this time the virus has not been identified. Results of IEM indicate this virus is related to the California subgroup of Toga viruses. Toga viruses and members of the Orbivirus subgroup of the Reoviridea have been implicated in bovine

abortion in Japan and Africa. (20,33,54) Lesions suggestion the presence of infection were seen in 7 of the 8 fetuses containing the 1721 virus, but none of the fetuses had Ig levels greater than the maximum normal level of 20 mg/dl. The 1721 virus was present in two fetuses that were aborted 1 month apart in the same herd.

Koch's postulates must be fulfilled in order to determine the significance of the 1721 virus as an abortifacient agent. When the virus has been characterized, and if it should prove abortifacient, screening methods need to be developed to determine the prevalence of antibody to the virus in the cattle population. The availibility of a rapid and specific test such as FAT to examine tissues from aborted fetuses for the presence of the virus would aid in determining the number of aborted fetuses infected with the virus. The effects of the virus on the fetus and the dam also should be investigated.

Considering the time and effort involved in preparing and examining specimens and the nature of the results obtained, it seems unlikely that routine EM examination of fetal tissues should become routine diagnostic procedure. Electron microscopic examination of tissues from fetuses that have extensive lesions or greater than normal Ig levels, and in which no infectious agent has been found, might reveal otherwise undisclosed infectious agents. If these agents prove to be abortifacient, other more practical means of diagnosis probably can be developed.

Summary

1. Negative stain EM was conducted on lung and liver tissues from 500 aborted calves received at the South Dakota Animal Disease Research and Diagnostic Laboratory.
2. Herpesvirus was observed in the tissues from 35 aborted bovine fetuses. In 30 of these cases, the virus was confirmed as IBR by FAT, and lesions typical of IBR infection were observed in the tissues of these fetuses. The identity of the other 5 herpesvirus-like particles was not established.
3. Coronavirus-like particles were observed in the tissues from 9 aborted bovine fetuses. These particles could not be grown in culture, and their identity was not established.
4. Objects resembling mycoplasma were observed in approximately 8% of the cases examined. All attempts to culture these objects failed.
5. A particle resembling reovirus was seen in tissues of one fetus, and one resembling influenzavirus was seen in tissues of another fetus. The identity of these particles was not established.
6. A dense fibrous matrix size was observed in the tissues from 8 aborted fetuses. This virus, designed 1721, was isolated using bovine turbinate cell culture. Immuno-electron microscopy indicated that this virus reacts with antiserum to the La Crosse strain of California encephalitis virus. At the time of this writing this virus has not been completely identified.
7. Before any significance can be attributed to the presence of this virus in the fetal tissues, Koch's postulates must be fulfilled.

8. Considering the difficulty of the procedure and the nature of the results obtained, it is doubtful that routine EM examination of fetal tissues has value in the diagnosis of bovine abortion.
9. Electron microscopy of tissues from fetuses in which infection is suspected but no infectious agent can be found might reveal otherwise undiscovered infectious agents.

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